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A novel resistance-linked ovine PrP variant and its equivalent mouse variant modulate the *in vitro* cell-free conversion of rPrP to PrP^{res}

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Summary

Prion diseases are associated with the conversion of the normal cellular prion protein, PrP^{c}, to the abnormal disease associated PrP^{Sc}. This conversion can be mimicked *in vitro* using the cell-free conversion assay. We have recently shown that this assay can be modified to use bacterial recombinant

PrP as substrate and mimic the *in vivo* transmission characteristics of rodent scrapie. Here we demonstrate that the assay replicates the ovine polymorphism barriers of scrapie transmission. In addition, the recently identified ovine PrP variant ARL^{168}Q, which is associated with survival of sheep to experimental BSE, modulates the cell-free conversion of ovine recombinant PrP to PrP^{res} by 3 different types of PrP^{Sc}, reducing conversion efficiencies to levels similar to the ovine resistance-associated ARR variant. Also, the equivalent variant in mice (L^{164}) is resistant to conversion by 87V scrapie. Together these results suggest a significant role for this position and/or amino acid in conversion.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases including Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats and bovine spongiform encephalopathy (BSE) in cattle. The causative agent responsible for TSEs or prion diseases has yet to be fully defined. However, a fundamental event in disease is the conversion of the normal, proteinase K (PK) sensitive isoform of the prion protein, PrP^{c}, to an abnormal, partially PK resistant isoform, PrP^{Sc}, and the accumulation of this abnormal isoform in the central nervous system of infected animals (Hope *et al*., 1986; Meyer *et al*., 1986; Oesch *et al*., 1985). Although the mechanism of conversion is unknown, interaction between PrP^{c} and PrP^{Sc} is critically implicated by *in vitro* studies (Caughey & Chesebro, 1997) and features in the current models of replication (Prusiner, 1991; Jarrett & Lansbury, 1993).

Polymorphisms in PrP are associated with susceptibility to and pathology of TSEs. The major determinants controlling the susceptibility of sheep to scrapie are

polymorphisms at PrP amino acid codons 136, 154 and 171 (Hunter *et al*., 1997). The PrP ARR allele (amino acids, in single letter code, at positions 136, 154 and 171 respectively) is associated with resistance to classical scrapie. The PrP ARQ and VRQ alleles are associated with susceptibility to disease. Novel ovine PrP polymorphisms are regularly being identified in sheep genotyping programs. Most of these novel polymorphisms occur with low frequency and their association with disease susceptibility is not known (Baylis & Goldmann, 2004). It is important to assess the disease association of such variants in the hope of identifying additional scrapie resistant alleles, as not all breeds or populations have significant frequencies of the known resistant ARR allele and it has been reported that sheep homozygous for the ARR allele may be susceptible to atypical scrapie (Bushmann *et al*., 2004) and intracerebral (ic) inoculation with BSE (Houston *et al*., 2003). The identification of other resistant alleles may also help to protect against novel strains of scrapie or adaptation to a particular genotype.

Due to the long incubation time and high cost of animal experiments, the *in vitro* cell-free conversion assay has provided a quick, well defined system in which to assess the disease association of such alleles (Kocisko *et al*., 1994). However, for natural infection, dose, route, strain of agent, influence of second allele and breed of sheep are all likely to play a role in TSE susceptibility and additional evidence from experimental challenge would be required to support any association of particular alleles with resistance to TSEs. In the cell-free conversion assay PrP^{Sc}, isolated from the brains of scrapie infected animals, induces the conversion of radiolabelled recombinant PrP, to a PK resistant isoform, PrP^{res} (Kocisko *et al*., 1994). The assay has been shown to replicate *in vivo* species specificity, strain properties and polymorphism barriers (Bessen *et al*., 1995; Bossers *et al*., 1997; Bossers *et al*.,

2000; Iniguez *et al*., 2000; Kocisko *et al*., 1995; Zhang *et al*., 2002; Raymond *et al.*, 1997; Horiuchi *et al*., 2000) and has been used to study many aspects of molecular conversion. As yet, however, no *in vitro* cell-free generated PrP^{res} has been shown to be infectious (Hill *et al*., 1999). Recently, we reported the use, as substrate, of mouse and hamster PrP, biochemically purified from recombinant bacteria and demonstrated that the assay replicated several characteristics of *in vivo* disease (Kirby *et al*., 2003).

Evidence is presented here that bacterial recombinant ovine PrP can be converted to PrP^{res} in the cell-free conversion assay and the sheep polymorphism barriers of scrapie transmission are replicated. Full length ovine PrP of the ARR, ARQ and VRQ genotypes, with the N-terminal signal sequence replaced with methionine and the C-terminal signal sequence removed, corresponding to amino acids 25-233, were PCR amplified from genomic DNA using the 5' and 3' primers, 5'-GGATCCATCATGAAGAAGCGACCAAAACCTGGC-3' and 5'-CCGAATTCTCATGCCCCCCTTTGGTAATAA-3', respectively. Plasmid pTrcHIS B (Invitrogen) was digested with restriction enzymes *Nco*I and *Eco*RI to remove the 6-histidine tag. PCR fragments were digested with restriction enzymes *Eco*RI and *Rca*I and ligated into the modified pTrcHis B plasmid. Therefore, the vector encodes full-length, untagged ovine PrP. Calcium chloride competent *Escherichia coli,* strain 1B392 (Wright *et al*., 1986), were transformed with the recombinant vectors. Ovine PrP variants were expressed, radiolabelled, purified, refolded and characterized by mass spectrometry and circular dichroism, as described in Kirby *et al*., (2003). A representative autoradiograph is shown in Figure 1, lanes 1-3. PrP^{Sc} was purified from the brain stems of VRQ homozygous sheep clinically infected with scrapie (SSBP/1 source), from ARQ homozygous sheep clinically infected with BSE and

101 from BSE infected cows, based on a method described by Hope *et al*., (1986). Cell-
102 free conversion assays were carried out using the 3 radiolabelled ovine PrP variants
103 (^{35}S-rARRPrP, ^{35}S-rARQPrP, and ^{35}S-rVRQPrP) as substrates and the 3 different
104 PrP^{Sc} types as seeds, as described previously (Kirby *et al*., 2003). Briefly, 1 µg of
105 PrP^{Sc} was incubated with 200 ng of ^{35}S-rPrP for 24 hours at 37°C, in a non-
106 guanidine containing conversion buffer. Following incubation, 1/20th of the reaction
107 was treated with 60 µg PK ml^{-1} for 1 hour at 37°C. PK digestion was stopped by
108 adding Pefabloc to 1 mM. All samples were methanol precipitated and analysied by
109 SDS-PAGE and autoradiography. Autoradiographs were quantifed using Phoretix
110 Gel Analysis Software. A typical autoradiograph is shown in Figure 1A. The
111 experiment was repeated 3 times, efficiencies of conversion determined by
112 densiometric analysis and the mean conversion efficiencies calculated (±SE) by
113 densiometric analysis of labeled PrP before and after PK treatment (Figure 1B).

114 Figure 1A shows that bacterial recombinant ovine PrP is converted into a PK
115 resistant form in the cell-free conversion assay. The conversion efficiencies obtained
116 using bacterial recombinant ovine PrP were low (Figure 1B) in comparison to the
117 efficiencies obtained using bacterial recombinant rodent PrP (Kirby *et al*., 2003).
118 Conversion efficiency may be increased by the addition of guanidine to the
119 conversion buffer (Bossers et al., 1997). Indeed, it has recently been reported that
120 the addition of guanidine to the assay buffer is essential to obtain any conversion of
121 sheep PrP (Piening *et al*., 2006). However, this reduces the physiological relevance
122 of the assay as a model of conversion. In addition, the conversion efficiencies
123 between sets of experiments (a set is defined as the 5 types of ^{35}S-rOvPrP and 1
124 type of PrP^{Sc}) varied enormously (Figure 1B). This has been reported by others
125 using a similar cell-free conversion assay (Bossers *et al*., 1997). The reason for this

is unknown, but breed of sheep or the area of brain selected for purification of PrP^{Sc} may contribute. However, the relative conversion efficiencies within a set were similar each time the experiment was repeated. Of the 3 frequently occurring ovine PrP variants, ARR, ARQ and VRQ, ^{35}S-rARRPrP, the variant associated with resistance to classical scrapie, consistently produced the lowest amount of PrP^{res} when used as substrate in the cell-free conversion reaction (Figure 1A, lanes 6, 11 and 16). ^{35}S-rARQPrP and ^{35}S-rVRQPrP, variants associated with susceptibility to scrapie and short incubation times, converted with higher efficiencies (Figure 1A, lanes 7, 8, 12, 13, 17 and 18). A switch in the convertibility of ^{35}S-rARQPrP and ^{35}S-rVRQPrP is evident with homologous and heterologous PrP^{Sc}. The homologous conversion reactions produced the greatest amount of PrP^{res} (Figure 1A, lanes 8 and 17). These results indicate that the known *in vivo* polymorphism barriers of scrapie transmission can be mimicked in the cell-free conversion assay, using bacterial recombinant ovine PrP.

Recently, Goldmann *et* al., (2005) have identified two novel ovine PrP alleles. Sheep carrying a PrP variant with a proline to leucine polymorphism at amino acid 168 (L^{168}) were shown to have increased survival time to experimental BSE in 2 independent experiments (Goldmann, **this reference refers to Wilfred Goldmann's paper to be published alongside my paper). $ARL^{168}Q$ occurs at low frequency and although it may be linked with resistance to experimental BSE no data exists on its resistance to scrapie infection. The other variant $ARQE^{175}$, with a change from a glutamine to a glutamic acid at amino acid position 175 (E^{175}) is also rare and not yet associated with scrapie or BSE susceptibility. Therefore, the cell-free conversion assay was used to predict whether these ovine variants are associated with resistance to scrapie infection. The 168 and 175 amino acid positions are of interest

151 as they are located close to the putative factor-X binding site (Telling *et al*., 1995;
152 Kaneko *et al*., 1997) and the resistance-associated R^{171} position.

153 In a further experiment ^{35}S-rARL^{168}QPrP and ^{35}S-rARQE^{175}PrP were
154 produced (Figure 1A, lanes 4 and 5), as described above for the other ovine
155 variants, and used as substrate in the cell-free conversion assay incubating with the
156 3 different PrP^{Sc} types. ^{35}S-rARL^{168}QPrP converted with low efficiency using all 3
157 types of PrP^{Sc} (Figure 1A, lanes 9, 14 and 19), indicating that the $ARL^{168}Q$ effect on
158 conversion is significant for scrapie as well as BSE. ^{35}S-rARQE^{175}PrP converted with
159 an efficiency similar to ^{35}S-rARQPrP with the 3 different types of PrP^{Sc} (Figure 1A,
160 lanes 10, 15 and 20), suggesting that this amino acid change does not affect
161 conversion. Table 1 provides a rank order of conversion efficiences of the ^{35}S-
162 rOvPrP variants with the 3 different PrP^{Sc} types.

163 Due to the low conversion efficiencies and to strengthen the data showing the
164 reduced conversion efficiency effect of the ovine ARL^{168}QPrP variant, it was
165 determined whether the equivalent site in mouse PrP would have the same effect
166 and reduce conversion efficiency. The mouse cell-free conversion assay gives
167 higher conversion efficiencies and therefore the effect on conversion can be more
168 easily seen. Also, polymorphisms associated with resistance to TSEs in both sheep
169 (R^{171}) and humans (K^{219}) have been engineered on a mouse background (R^{167} and
170 K^{218}, respectively) and the recombinant versions shown not to convert when
171 expressed in ScN2a cells (Kaneko *et al*., 1997), suggesting that mouse PrP can be
172 used to model the effects of PrP polymorphisms in other species.

173 The murine equivalent of ovine amino acid 168 is 164. Full length mouse PrP
174 (amino acids 23-230) of the *Prn-p*^{a} genotype with a proline to leucine mutation at
175 amino acid 164 was constructed by site directed mutatgenesis (QuickChange II,

Stratagene) using the full length mouse PrP clone, the production of which has been described previously (Kirby *et al*., 2003), as a template and the following primers; 5'-CCAAGTGTACTACAGGCTAGTGGATCAGTACAGC-3' and 5'-GCTGTACTGATCCACTAGCCTGTAGTACACTTGG-3'. Rosetta *Escherichia coli* (Novagen), which over expresses the rare leucine tRNA, were transformed with pTrcMoL^{164}PrP. MoL^{164}PrP and MoPrP were expressed, radiolabelled, purified and characterized as described for the ovine variants. PrP^{Sc} was purified from the brains of terminally ill 87V-infected VM mice, based on a method described by Hope *et al.,* (1986). Cell-free conversion assays, using the 2 mouse PrP variants as substrates and 87V PrP^{Sc} as seed, were carried out in the absence of guanidine and analysed as described previously (Kirby *et al*., 2003). The experiment was repeated at least 3 times and a typical autoradiograph is shown in Figure 2.

Figure 2 shows that ^{35}S-rMoPrP is efficiently converted to PrP^{res} in the presence of 87V PrP^{Sc} (Figure 2, lane 2). However, ^{35}S-rMoL^{164} PrP is not converted to PrP^{res} in the presence of 87V PrP^{Sc} (Figure 2, lane 3), suggesting that this amino acid/position may be a significant site in the conversion process in other species.

It is not understood how substitution of different amino acids at certain positions within PrP has such a profound effect on susceptibility. It has been suggested that mutations can modulate the stability of PrP^{c}, PrP^{Sc}, or both, or can affect the binding of PrP to effector molecules. In the case of a proline to leucine mutation, the amino acids share similar hydrophobicity, but are structurally diverse. Proline, the only imino acid, has backbone torsion angles that are tightly controlled as a result of its cyclic structure. Because of this, it results in turns in protein backbones and often occurs at the end of β-sheets. A change from proline to leucine, which has a greater range of flexibility in its backbone angles, may reduce

the propensity of PrP to form β-sheets, and explain the significant protective effect of this mutation. Alternatively, leucine is an amino acid capable of involvement in many types of secondary structure, including α-helices, and a change to a leucine may aid the stabilisation of PrP^{c}. Interestingly, the same mutation, proline to leucine, is associated with apparent spontaneous disease in humans when it occurs at position 102 in human PrP, the reverse of what we find for ovine position 168 and its equivalent mouse position. This is almost undoubtedly a reflection of the very different tertiary structures in the different parts of the molecule and the different involvement of these areas in conversion of PrP^{c} to PrP^{Sc}. In addition, extensive gene-targeted transgenic mouse experiments show that L^{101} mice have altered susceptibility to a range of TSE isolates compared to wildtype mice (Barron *et al*., 2001), further complicating interpretation. The neutral phenotype of the ovine PrP E^{175} polymorphism in our assay suggests that not every change in this region of PrP will affect susceptibility and indicates that the underlying mechanism may be highly positional and residue specific.

To determine the molecular mechanisms responsible for the protective effect of the L^{168} mutation, substitution of a range of different amino acids at position 168 would be required and their affect on conversion assessed. Such experiments are currently underway in our laboratory using the murine cell-free conversion assay as a model. In addition, the ovine and murine version of the cell-free conversion assay using bacterial recombinant PrP can be used to assess the link between novel ovine polymorphisms, as they are identified, to classical scrapie, atypical scrapie, such as NOR98, and to BSE.

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Figure 1 legend:

A) An autoradiograph of a cell-free conversion reaction using 5 ovine recombinant variants (^{35}S-rOvPrP) as substrate and PrP^{Sc}, isolated from scrapie infected VRQ/VRQ homozygous sheep brains (lanes 6-10), BSE infected cow brains (lanes 11-15) and BSE infected ARQ/ARQ homozygous sheep brains (lanes 16-20) as seeds. Lanes 6-20 have been exposed for a longer time interval than lanes 1-5 in order to detect PrP^{res}. Molecular mass markers are indicated on the left in kDa. (PK) proteinase K. Boxed area indicates PrP^{res}. **B)** Average conversion efficiencies (±SE) for each set of conversion reactions (5 types of ^{35}S-rOvPrP and 1 type of PrP^{Sc}).

Table 1 legend:

A summary table of sheep conversion assay data. Each set of conversions (5 ^{35}S-rOvPrP variants converted by 1 type of PrP^{Sc}) are ranked in order of decreasing conversion efficiency, predicting the susceptibility of the ovine variants to scrapie and BSE.

Figure 2 legend:

An autoradiograph of a cell-free conversion reaction using mouse PrP with and without a proline to leucine mutation at amino acid position 164 (equivalent to ovine position 168) as substrates and 87V PrP^{Sc} as seed. Molecular mass markers are indicated on the left in kDa. (PK) proteinase K. Boxed area indicates PrP^{res}.

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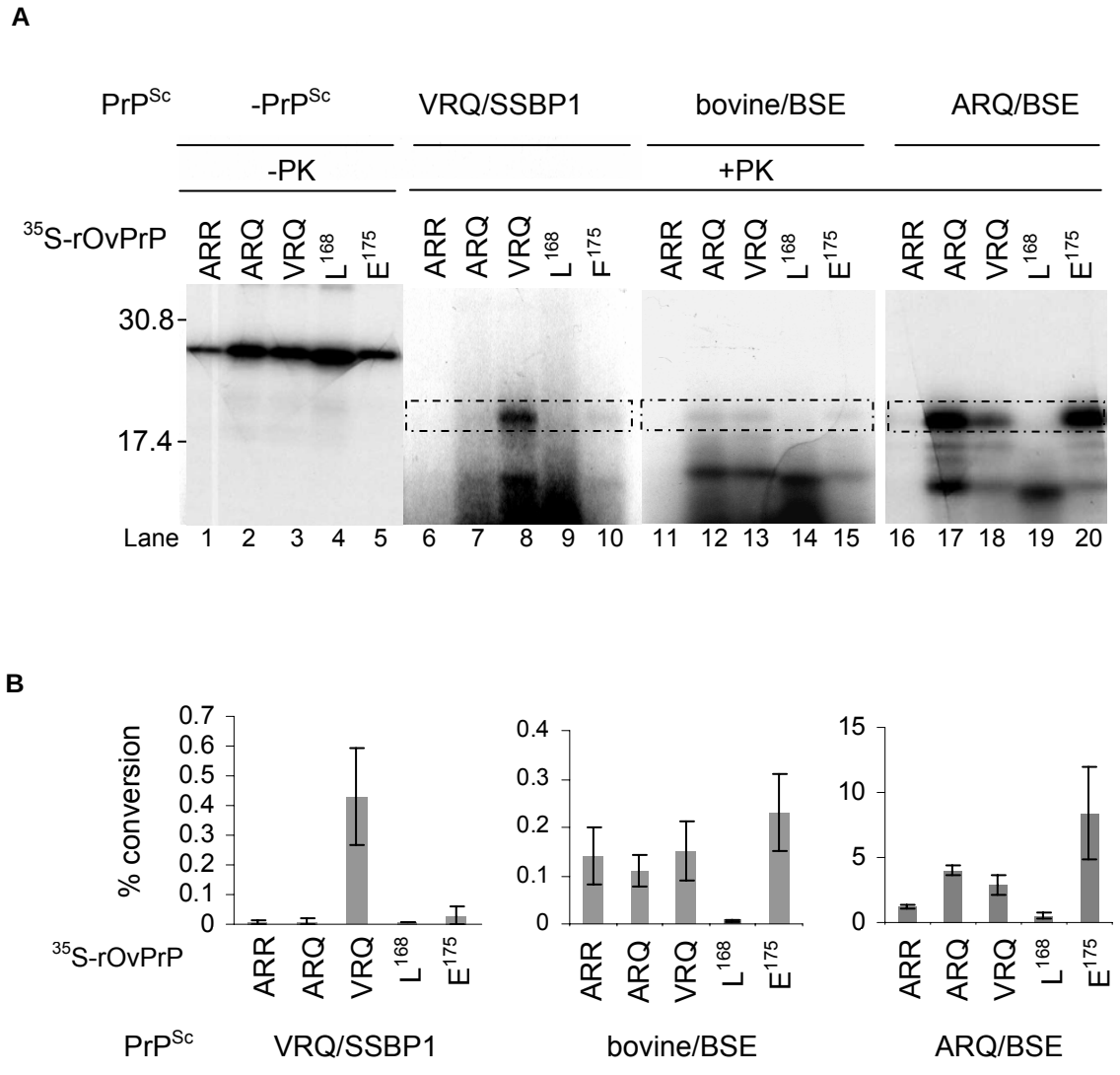
Figure 1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PrP^Sc
-PrP^Sc
VRQ/SSBP1
bovine/BSE
ARQ/BSE
-PK
+PK
35S-rOvPrP
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ARR ARQ VRQ L168 F175
ARR ARQ VRQ L168 E175
ARR ARQ VRQ L168 E175
30.8
17.4
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
% conversion
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
35S-rOvPrP
ARR ARQ VRQ L168 E175
0.4
0.3
0.2
0.1
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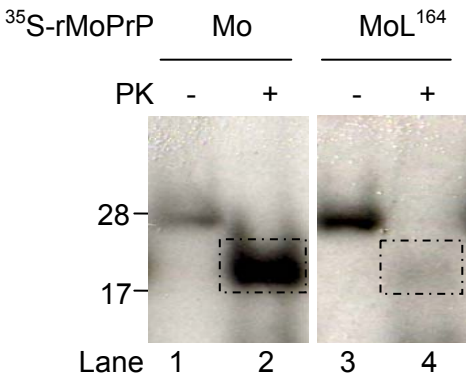
15
10
5
0
PrP^Sc
VRQ/SSBP1
bovine/BSE
ARQ/BSE

366

Figure 2



367 **Table 1**

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369

370

PrP^{Sc}	Conversion efficiency of ^{35}S-rOvPrP High ----------► Low				
VRQ/SSBP1	VRQ	$ARQE^{175}$	ARQ	ARR	$ARL^{168}Q$
Bovine/BSE	$ARQE^{175}$	VRQ	ARR	ARQ	$ARL^{168}Q$
ARQ/BSE	$ARQE^{175}$	ARQ	VRQ	ARR	$ARL^{168}Q$
consensus	ARQ/$ARQE^{175}$		VRQ		ARR/$ARL^{168}Q$

371